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HORMONAL CONTROL OF REPRODUCTION IN *BUSYCON*: LAYING OF EGG CAPSULES CAUSED BY NERVOUS SYSTEM EXTRACTS

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Hormones control many aspects of reproductive behavior and the functioning of reproductive organs in gastropods. In prosobranch gastropods, factors controlling sex reversal, which occurs in some species, and sex organ development have been studied (Le Gall and Streiff, 1975). Neurohormonal factors causing egg-laying behavior have been found in opisthobranch gastropods, such as *Aplysia*, (Arch, 1976, for review) and *Pleurobranchaea* (Davis, Mpitsos, and Pinneo, 1974; Ram, Salpeter, and Davis, 1976), and the pulmonate gastropod *Lymnaea* (Geraerts and Bohlken, 1976), but they have not previously been reported in prosobranchs. To provide a broader taxonomic and functional basis on which to make comparative observations, experiments were undertaken on the dioecious prosobranch *Busycon* to determine whether neurohormones controlling egg-laying behavior were to be found in prosobranchs, and also to investigate their presence in a dioecious animal, since *Aplysia*, *Pleurobranchaea*, and *Lymnea* are all hermaphrodites.

Busycon is the largest marine gastropod found along the eastern coast of the United States. In Woods Hole, Massachusetts, two species of *Busycon* are found: *B. canaliculatum*, the channeled whelk, and *B. carica*, the knobbed whelk. *Busycon* lays its eggs encased in disciform capsules, which are attached to one another in long strings. The capsules laid by the two species of *Busycon* have different shapes, with *B. carica* laying capsules with smooth sides and *B. canaliculatum* laying capsules with ribbed sides (Magalhaes, 1948).

The behavior involved in forming and laying the egg capsules is very similar to the behavior described by Ankel (1929) for *Nassarius*, another dioecious prosobranch. Briefly, a soft bulb-shaped egg capsule is passed from the female gonopore through a groove in the side of the foot to a gland in the bottom of the foot known as the pedal pore. The capsule is hardened and given its species specific shape in the pedal pore, and at the same time the capsule is glued to the substrate or to a previously laid egg capsule to extend the string.

A preliminary report of some of these data has been published (Ram, 1975).

Materials and Methods

All work reported here was done at the Marine Biological Laboratory (MBL), Woods Hole, Massachusetts from July to September, 1975. Specimens of *Busycon canaliculatum* and *Busycon carica* were collected locally by the MBL Supply Department and maintained in individual (27 cm × 16 cm × 10 cm deep) or group

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aquaria with running sea water at the ambient temperature of 20–25° C. Animals were individually numbered. At the time of dissection, animals were weighed with and without shell; the sexual maturity was estimated by weighing the gonad (Strumwasser, Jacklet, and Alvarez, 1969; Betzer and Pilson, 1974) and calculating the gonad index, *i.e.*, gonad weight/animal weight without shell (Betzer and Pilson, 1974). Animals were generally not sexed until the time of dissection; however, it was usually possible to select females when needed, since the largest animals were almost always females: for *B. canaliculatum* 98% (N = 52) over 300 g (weight with shell) were female, 79% (N = 24) 250–300 g were female, and 55% (N = 36) under 250 g were female.

To dissect the nervous system, the animal's shell was removed and the central nervous system, except the visceral ganglion, was exposed by cutting through the side of the foot along the line of attachment of the mantle. Several red-pigmented ganglia surround the esophagus (Pierce, 1950). [Abbreviations used in this paper to identify parts of the nervous system are: V, visceral ganglion; CNS, central nervous system not including V; and CNR, CNS minus buccal and pedal ganglia.] A short piece of esophagus with the CNS attached was removed, and connective tissue covering the nervous system was dissected from the CNS. The CNS, including a short piece of esophagus, or individual ganglia, was then homogenized in a motor-driven glass-glass tissue grinder in a bed of ice. When needed, the visceral ganglion was dissected from the thin mantle tissue joining the gills to the rectum.

Tissue was first homogenized as dissected for 30 sec. Next, 1.0 ml phosphate buffer (sodium salt, 0.2 M, pH 7.1) or filtered sea water was added, and the sample was homogenized an additional 30 sec. In most experiments the homogenate was centrifuged at 350–500 × *g* for two min in a hand-driven centrifuge, and the supernatant was decanted. The supernatant was diluted as required with an additional amount of the medium used to homogenize, and 0.5 ml aliquots were drawn into disposable 1.0 ml syringes.

Animals were injected through the side of the foot adjacent to the siphon end of the operculum using a 25 g 0.625 inch needle, which was pushed its full length into the side of the foot. Injections of dye by this route showed it to be effective for injecting fluids into the pedal sinus. In most instances, particular samples were tested on at least two recipients in order to increase the reliability with which the hormone, if present, would be detected.

Modifications of the above procedures, to investigate the active agent's solubility and stability to boiling, are described in the results. To study the sensitivity of the agent to protease, parietal ganglia from several animals were homogenized in filtered sea water to yield five or more parietal ganglia/ml. The homogenate was put on a boiling water bath for ten minutes and then cooled on ice for five minutes. This boiling step was necessary because preliminary experiments showed that activity in a crude, unboiled homogenate was lost in five min at 37° C, presumably as a result of degradation by enzymes in the crude extract. After cooling on ice, the homogenate was centrifuged at 350–500 × *g* for two min, and 0.3 ml aliquots were removed from the supernatant for further treatment. The experimental aliquot was preincubated for five min at 37° C, and then 0.3 ml of protease solution (Protease VI, Sigma from *Streptomyces griseus*, 0.01 mg/ml of sea water, preincubated five min at 37° C) was added. After ten min incubation,

the sample was put on a boiling water bath for five min to destroy the protease activity. After cooling, 0.5 ml of filtered sea water was added, and the sample was injected into two recipients. Control aliquots, with sea water instead of protease solution added and either incubated as usual (incubated control) or not incubated at all (cold control), were tested. A control for injection of boiled protease in the experimental extract was done by adding boiled protease solution to an active extract prior to injection (inactive protease control).

Results

Induction of egg-laying behavior by nervous system extracts

Laying of egg capsules could be induced by injection of nervous system extracts of *Busycon* into recipient *Busycon*. Both conspecific and interspecific (between *B. carica* and *B. canaliculatum*) injections were effective. The first injection produced the laying of egg capsules in 15 (40%) out of 38 female recipients (Table I). Fifteen of the 23 animals which failed to lay egg capsules on the first injection were tested on subsequent occasions, and, of these, three animals (20%) laid egg capsules.

Animals which had laid egg capsules at least once nearly always laid egg capsules upon subsequent injections (Table II). Injections of CNS or CNR extracts into these animals caused egg capsule laying on 27 (80%) out of 34 occasions. Only one animal which had laid egg capsules once could not be induced to lay on subsequent occasions. (This animal accounts for two of the failures.) Except where otherwise stated, all of the results described were obtained using animals which had been induced to lay egg capsules at least twice. Control injections of phosphate buffer or sea water into these animals never resulted in egg capsule laying (Table II).

To determine whether the difference between animals which reliably laid egg capsules and those which never laid might be attributed to lack of sexual maturity of the nonlayers, the relationship between egg capsule laying and gonad size was examined. Of the specimens of *B. canaliculatum* tested, six had a gonad weight below 0.9 g and a gonad index less than 0.006, and all six failed to lay egg capsules. Five of these had been tested with extracts which caused at least one other animal to lay capsules. Animals with a gonad weight or gonad index above these figures had a greater than even chance of being reliable egg layers [14 (58%) layers out

Table I

Laying of egg capsules after first injection with nervous system homogenate (CNS or CNS + V).

Donor species	Recipient species	Animals laying/animals injected		
		Homogenizing buffer		
		PO_4	Sea water	Total
B. canaliculatum	*B. canaliculatum*	9/24	2/6	11/30
B. canaliculatum	*B. carica*	1/3	0/1	1/4
B. carica	*B. canaliculatum*	0/0	3/4	3/4
Total		10/27	5/11	15/38

TABLE II

Laying of egg capsules by "reliable layers" (animals which had laid egg capsules on at least one previous occasion).

Part of nervous system injected	Animals laying/animals injected		
	Homogenizing buffer		
	PO_4	Sea water	Total
CNS + V	6/7	0/0	6/7
CNS	11/14	1/1	12/15
CNR	2/2	7/10	9/12
Total	19/23	8/11	27/34*
Homogenizing buffer alone	0/10	0/6	0/16

* Includes 7/9 injections of *B. canaliculatum* into *B. carica* and 2/2 injections of *B. carica* into *B. canaliculatum*. All others are *B. canaliculatum* into *B. canaliculatum*. These are the results of injections into 12 recipient animals.

of 24 animals for which data are available, Fig. 1]. Five of the ten nonlayers in this group had been tested with extracts which caused other animals to lay egg capsules. Although animals with smaller gonads were thus unlikely to lay egg capsules when injected with active extracts, gonad size was not the sole determinant of capsule laying, since not all animals with large gonads could be induced to lay egg capsules.

The egg capsules laid as a result of extract injection were unusual in three respects. First, capsules laid after extract injection did not contain any eggs,

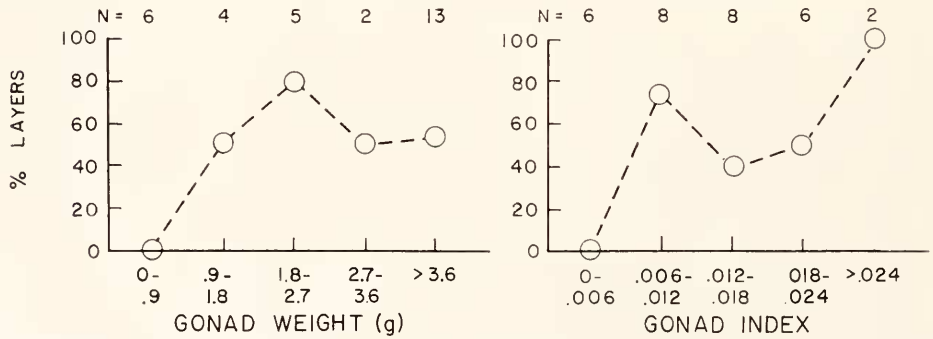

FIGURE 1. Relationship between gonad size and incidence of egg laying of animals injected with nervous system extracts. The graphs illustrate the relationship using two different measurements of gonad size: gonad weight and gonad index (gonad weight/animal weight without shell). Graphs show percentages of animals in various ranges of gonad weight or index which laid egg capsules at least twice. N is the total number of animals in the indicated weight or index range. Data illustrated are from all animals for which complete data were collected, except one animal which laid once and could not be induced to lay again. This animal was at the lower end of the layer distribution (gonad weight = 0.85 g, gonad index = 0.0051).

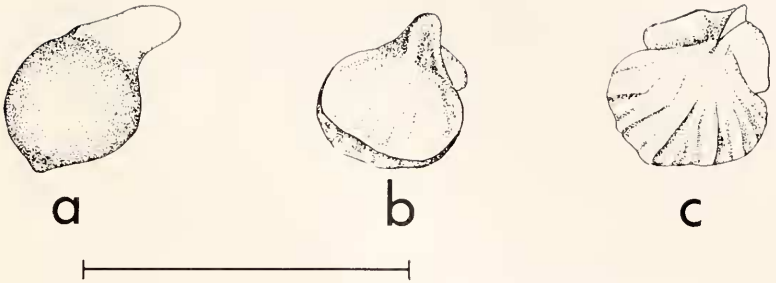

FIGURE 2. Egg capsules laid as a result of nervous system extract injections: (a) soft, bulb-shaped capsule; (b) hardened, well-formed capsule with relatively smooth sides and a double edge laid by *B. carica* injected with *B. canaliculatum* extract; (c) hardened, well-formed capsule with ribbed sides and a single edge laid by *B. canaliculatum* injected with *B. canaliculatum* extract. Similarly shaped capsules were laid by *B. canaliculatum* injected with *B. carica* extract. Scale equals 5 cm.

except in one instance to be discussed later. The active agent will therefore be referred to only as an egg capsule laying substance (ECLS), and not as an egg-laying hormone. Secondly, the capsules often were not well formed and hardened, as spontaneously laid egg capsules usually are. Thirdly, the capsules were, except in one instance, laid individually and were not strung together in strings as are found in the wild.

Figure 2 shows examples of egg capsules laid as a result of extract injections. Most of these egg capsules were soft and bulb-like in appearance (Fig. 2a). Occasionally reasonably well-formed and hardened egg capsules were laid (Fig. 2b, c). The success of the animal in passing the capsule over the edge of the foot into the pedal pore determined whether a soft or hard capsule was produced. On most occasions the capsule slipped out of the groove in the side of the foot, and the resultant capsule was soft and bulb-like. If the capsule was successfully passed to the pedal pore, it was enveloped by the pore for 15 minutes to several hours, after which it emerged as a hardened, well-formed capsule. If a hardened capsule was laid, its shape was typical of the species laying it, regardless of the donor extract (Fig. 2b, c).

The latency between extract injections and the laying of an egg capsule was about two to four hours. If the laying of more than one egg capsule was induced, subsequent capsules appeared at a spacing of about one every three hours.

The usual dosage in these experiments was one-quarter or one-half of the extract of a single CNS. Capsule laying was induced by as little as one-eighth of a CNS extract; however, this was the lowest dose tested, and no systematic investigation of the lowest effective dose was attempted. Injection of greater dosages or several injections of one-quarter CNS dosages, spaced over several hours, frequently resulted in several capsules being laid. The most extreme example of this was a single injection of one CNS + V, which resulted in the laying of six unattached egg capsules over about half a day. Injection of an animal with one-quarter CNS dosages at approximately three hour intervals usually caused an egg capsule to be laid about three hours after each injection. Moreover, injection of extract on the following day would also cause egg capsule laying.

Thus, there appeared to be no refractory period in *Busycon* beyond the time necessary to induce a single egg capsule. This made it possible to use animals repeatedly, though recipients were generally given only one injection per day.

Localization of ECLS within the CNS

ECLS was localized within the nervous system by dissecting parts of the nervous system. In the first set of experiments the usual centrifuged extracts in phosphate buffer were made; in later experiments uncentrifuged sea water extracts were injected. Extracts equivalent to either one-quarter or one-half of the dissected tissue in a whole animal were injected, usually into each of two recipients for each sample. Recipients were chosen by lot, and the identity of injected extracts was concealed until several recipients had laid egg capsules.

In the first series of experiments, with centrifuged phosphate buffer extracts, the parietal ganglia gave the most consistent positive results; seven (100%) out of seven extracts caused the laying of egg capsules. Less consistently, samples containing both the cerebral and pleural ganglia (the demarcation between these ganglia was indistinct, and they were therefore dissected together in this series) also caused the laying of egg capsules [three (60%) out of five extracts]. Extracts containing pedal, buccal or visceral ganglia, or a piece of esophagus did not cause the laying of egg capsules. The results are summarized in Table III.

It seemed possible that laying from cerebral-pleural samples might be caused by contamination by ECLS released during dissection. Therefore, in one of the above experiments, after removing the CNS from the animal, the CNS was frozen under 70% ethylene glycol (Giller and Schwartz, 1971) for further dissection of individual ganglia. The experiment still resulted in both the cerebral-pleural and parietal samples causing the laying of egg-capsules.

It was later discovered (see section on biochemical properties) that a considerable amount of activity was found even in the low speed pellet which was dis-

TABLE III

Localization of egg capsule laying substance within the nervous system (each extract tested on one or two recipients).

	Type of homogenate			
	PO_4		Sea water, uncentrifuged	
Part of nervous system tested	A	B	A	B
Parietals	7/7	13/14	6/6	10/10
Cerebrals and pleurals	3/5	5/10	—	—
Cerebrals and left pleural	—	—	4/4	4/4
Right pleural	—	—	4/4	4/5
Pedals	0/5	0/10	2/8	3/16
Buccals	0/5	0/7	—	—
Visceral	0/6	0/10	—	—
Esophagus	0/2	0/4	—	—

A: laying in one or more recipients / extract.
B: animals laying / animals injected.

carded in the above experiments. Therefore, a partial repetition of the localization experiment was done with uncentrifuged seawater extracts. In this set of experiments, the parietal ganglia, the cerebro-pleural ganglia, and one of the pleural ganglia all gave consistent positive results. Pedal ganglia extracts also occasionally gave positive results (Table III). The other ganglia were not tested in this series.

Presence of ECLS in males and females

To test whether ECLS was specific only to the female sex, male and female nervous systems were bioassayed. The CNS was dissected from large males [mean weight with shells = 219 g ± 13 g (s.d.)], and from females of about the same size [mean weight with shells = 210 g ± 18 g (s.d.)]. Extracts were made of individual nervous systems, and the equivalent of one-half of each CNS was injected into each of one or two recipients. On any particular day an equal number of male and female extracts were tested. Recipients were chosen by lot, and the identity of the injected extracts was concealed until several recipients had laid egg capsules.

The results of these experiments were that three (50%) out of six male extracts caused egg capsule laying, and five (83%) out of six female extracts caused egg capsule laying. In other experiments, without the parallel female controls, three out of three male extracts caused egg capsule laying.

Biochemical properties

To gain a better understanding of the ECLS and to provide possible guides for its future purification, experiments were performed on the heat stability, solubility, and protease sensitivity of the agent.

For heat stability, homogenates of CNR in filtered sea water were put on a boiling water bath for five minutes or thirteen minutes or were kept on ice as a control. Samples were subsequently centrifuged at 350–500 × *g* for two min, and the supernatants were injected into two recipients each. Both boiled extracts caused capsule laying (3/4 recipients), as did controls for each (3/4 recipients). In addition to these positive results, boiled extracts also consistently showed capsule laying activity in the experiments reported below on solubility and protease sensitivity.

For solubility, low speed (350–500 × *g* for two min) supernatants of CNR homogenized in 0.1 M phosphate buffer were subjected to 105,000 × *g* for two hours at < 4° C. No ECLS was found in the final supernatant (0/2 extracts, 0/4 recipients), whereas the 105,000 × *g* pellet contained ECLS (2/2 extracts, 3/3 recipients). Attempts to release ECLS from the 105,000 × *g* pellet by freeze-thawing or by exposing it to high salt concentrations (1.0 M NaCl for two hours at 0° C) failed; however, boiling the low speed supernatant of a sea water homogenate for ten minutes before centrifugation at 105,000 × *g* partially solubilized ECLS (one extract: 2/2 recipients of supernatant, 2/2 recipients of pellet).

The partition of the capsule-laying agent in the initial low speed (350–500 × *g* for two min) centrifugation was also studied. In three experiments using CNR extracts in filtered sea water, capsule laying was produced by three out of three pellets and two out of three supernatants.

Two extracts were tested for the protease sensitivity of ECLS. Samples treated with active protease never caused capsule laying (0/4 recipients), whereas all three control groups caused capsule laying (incubated control, 3/4 recipients; cold control, 4/4 recipients; inactive protease control, 4/4 recipients).

Spontaneous egg-laying

Spontaneous laying of egg capsules was observed on only one occasion. Since the conditions in which this occurred were unique for this laboratory, a description of these conditions may be a possible guide to future investigations of the natural controls of egg laying in *Busycon*.

Animals were usually maintained in individual aquaria; however, on August 29 the reliable layers in the laboratory (15 animals at the time) were put into a large group tank, along with several untested females and three males. When next observed, on September 3, several egg capsules had been spontaneously laid in the tank. Two animals had strings of egg capsules still attached to the foot. These two animals were transferred to individual aquaria. One of them continued to lay egg capsules until a string containing 79 egg capsules in all had been laid. This animal took more than a week to lay its capsule string, laying capsules at a rate of one capsule every three hours, a rate comparable to that seen in the field (Magalhaes, 1948). Capsule laying probably began about midnight on August 30, about 24 hours after the animals had been put in the group tank. The other animal which had an attached capsule string laid only one more capsule (for a total of 14 capsules) after being put into its own aquarium. This animal probably began laying about 48 hours after being put in the group tank. These capsule strings, laid in the laboratory, were identical in appearance to those usually found in the field (illustrated, for example, in Hyman, 1967, p. 304).

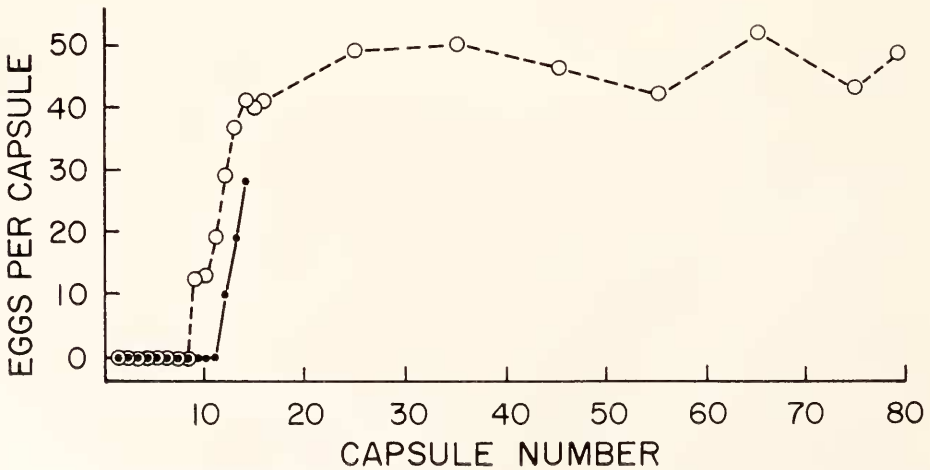

FIGURE 3. Number of eggs in selected capsules of two egg capsule strings laid in the laboratory. Open circles show data from a 79-capsule string; solid circles, data from a 14-capsule string.

The day after removing the two animals with attached capsule strings from the group tank, more spontaneously-laid capsules were found in the tank. At this point, all animals were separated into individual aquaria, and no more capsules were laid spontaneously.

The capsules in the 79-capsule and 14-capsule strings contained eggs. The number of eggs per capsule was counted by cutting open selected capsules in these two strings. These data (Fig. 3) show that the first 10 or so capsules in each string contained no eggs, and then the number of eggs gradually increased until, in the 79-capsule string, the number of eggs per capsule leveled off at about 45. Other strings of spontaneously-laid egg capsules, up to six capsules in length, contained no eggs.

The animal which laid the 79-capsule string was injected with nervous system extracts approximately 3 hours, 8 hours, 11 hours, and 21 hours after the last spontaneously-laid egg capsule was released from the foot. Each of these injections was followed by the laying of a single egg capsule containing eggs. These capsules had 9, 29, not recorded, and 8 eggs in them, respectively.

Discussion

Extracts of the nervous system of *Busycon* contain a substance (ECLS) which causes the laying of egg capsules when injected into female *Busycon,* demonstrating for the first time an agent causing such behavior in a prosobranch gastropod. ECLS may be a hormone which normally causes the laying of egg capsules; however, proof of this would require the demonstration of physiological concentrations of ECLS in the blood during normal laying of egg capsules. Like the egg-laying hormones of the opisthobranchs *Aplysia* (Toevs and Brackenbury, 1969) and *Pleurobranchaea* (Ram, Salpeter, and Davis, 1976), ECLS is sensitive to proteolytic enzymes, and therefore likely to be a polypeptide. It is also similar to the egg-laying hormones of *Aplysia* (Kupfermann, 1970) and *Pleurobranchaea* (Ram, Salpeter, and Davis, 1976) in being stable to boiling.

In contrast to the egg-laying hormones of *Aplysia* and *Pleurobranchaea,* which cause the laying of egg ribbons with eggs, ECLS usually causes the laying of egg capsules which do not contain eggs. It may be that ECLS causes the insertion of eggs into egg capsules, but only when it has been working over longer periods than were used in these experiments. The lack of eggs in the first 8–11 capsules laid by spontaneous layers (Fig. 3) may similarly be explained if ECLS must be secreted for up to a day before egg-containing capsules can be laid. Consistent with this explanation is the observation that ECLS caused laying of egg-containing capsules when injected into an animal that had already been laying eggs for several days.

Other explanations for the lack of eggs in capsules laid after ECLS injection are possible. For example, another agent, not found in the central nervous system, may be necessary. Perhaps eggs cannot be inserted unless they are first fertilized, as may have happened in the spontaneous layers which had been placed originally in a group tank. It is fairly evident, however, that lack of maturity of recipients is not an explanation for the lack of eggs. The gonad indices of the two animals which spontaneously laid capsules with eggs were 0.0096 and 0.0181, which is in the middle of the range of animals laying capsules (0.006 to 0.025, Fig. 1).

As in similar studies, on *Aplysia* (Strumwasser *et al.,* 1969), *Pleurobranchaea* (Ram, Salpeter, and Davis, 1977) and *Lymnaea* (Geraerts and Bohlken, 1976), not all animals could be induced to lay eggs or egg capsules. Fluctuations in the amount of hormone in the injected extract in *Aplysia* and *Pleurobranchaea* could not be eliminated as an explanation in those studies, because active extracts were never tested systematically on several recipients. In the present study, all but six of the nonlayers were tested with extracts that caused laying in at least one other recipient. Thus, factors within the recipient are responsible for this lack of response. Geraerts and Bohlken (1976) reached a similar conclusion in *Lymnaea.* In *Busycon,* one of these factors is gonad size. All six animals with gonad indices below 0.006 failed to lay capsules when injected with extract known to be active. Other factors, presently unknown, prevent some animals with gonad indices above this size from laying capsules.

Experiments on the localization of ECLS-containing cells indicate that the hormone was most consistently found in the parietal ganglia, but was also found in the cerebral and pleural ganglia. ECLS thus appears to be more widely distributed throughout the nervous system than the egg-laying hormone of *Pleurobranchaea* (Ram, Salpeter, and Davis, 1976), *Aplysia* (Strumwasser *et al.,* 1969), and *Lymnaea* (Geraerts and Bohlken, 1976, and Geraerts, personal communication). ECLS-synthesizing cell bodies may not be as widely distributed as this, since the hormone in some ganglia may be contained in processes which have their cell bodies elsewhere. The parietal ganglia, which gave the most consistently positive results, would seem to be the most likely site for the cell bodies producing ECLS. Hoffmann (1936) indicates that the bag cells of *Aplysia,* which synthesize its egg-laying hormone (Arch, 1976), are homologous to parietal ganglia; and Ram, Salpeter, and Davis (1977) propose that cells in the pedal ganglia of *Pleurobranchaea* which contain its egg-laying hormone are also homologous to parietal ganglia. Thus, the hormones in these animals and the cells synthesizing them may be homologous.

Similarities in some biochemical properties (stability to boiling and sensitivity to protease) have already been noted. ECLS appears to be less soluble than the egg-laying hormones of either *Aplysia* or *Pleurobranchaea;* however, even the latter appears to lose some activity into a low speed pellet (Ram, unpublished data). The insolubility of ECLS prior to boiling may be artifactual, but a more interesting possibility is that the hormone may be bound in vesicles or to a neurophysin which is denatured by boiling. Whatever the function of this insolubility for *Busycon,* it appears useful in purifying the hormone. Soluble proteins can be removed prior to boiling, and boiling can then be used to solubilize ECLS and probably a few other proteins.

ECLS was found in the nervous system of both male and female *Busycon.* While these experiments do not prove that the male ECLS is identical to the female ECLS, the presence in males of a hormone which in females controls a female function, such as egg-laying, is not unusual. In the octopus, the optic glands contain a hormone, which is identical in both sexes, and which stimulates vitellogenesis in females and spermatogenesis in males (Richard, 1970). In both the starfish (Kanatani and Oliguri, 1966) and the polychaete *Arenicola* (Howie, 1966), maturation of eggs and egg-laying are caused by agents found in both sexes. Prolactin is known to have many functions besides promoting mammary growth

and milk secretion in female mammals, for which it was named, and it occurs in both sexes of mammals as well as in amphibians, birds, reptiles, and teleost fish (Bern and Nicoll, 1968). Thus, it is a general principle that these "female" hormones occur in both sexes, and the discovery of ECLS in both sexes of *Busycon* supports this principle. The role of ECLS in male *Busycon* is unknown.

Further study is needed on the natural controls of egg-laying in *Busycon* and other gastropods. While it is known that a certain degree of sexual maturity must be attained before egg-laying behavior can be induced by hormone injection, it is not known why some seemingly mature animals cannot be induced to show any egg-laying behavior (Fig. 1, and similar analyses by Strumwasser *et al.*, 1969; Ram, Salpeter, and Davis, 1977). Moreover, environmental stimuli which are immediate causes of egg-laying in these animals are unknown. The almost simultaneous initiation of "spontaneous" egg-laying in *Busycon* when put in a group tank and its cessation upon separating the animals suggest that an analysis in *Busycon* may be possible.

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Summary

Mature specimens of female *Busycon* laid egg capsules when injected with extracts of nervous systems of male or female *Busycon*. The substance causing this behavior, named egg capsule laying substance (ECLS), was found most reliably in parietal ganglia, less consistently in cerebral-pleural ganglia, and rarely in other ganglia. Both species of *Busycon* found in Woods Hole, *B. canaliculatum* and *B. carica*, contained ECLS, and ECLS of each species was active in the other.

ECLS activity was not destroyed by boiling for up to fifteen minutes. Centrifugation of nervous system extracts at $105{,}000 \times g$ yielded ECLS only in the pellet. ECLS was not released from the pellet by freeze-thawing or by 1.0 M NaCl, but could be partially solubilized by boiling extracts before centrifugation. ECLS activity was destroyed by protease.

Several animals "spontaneously" laid strings of egg capsules after being put in a group tank with males and other females. Approximately the first ten capsules laid by these animals were devoid of eggs, after which egg-containing capsules were laid. Injection of ECLS into a spontaneous egg layer within a few hours after cessation of spontaneous egg laying caused the laying of capsules containing eggs. The possibility that ECLS may normally be responsible for the laying of both eggs and egg capsules is discussed.

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